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RESEARCH PAPER

Knockdown of the small conductance Ca^{2+} -activated K^{+} channels is potently cytotoxic in breast cancer cell lines

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BACKGROUND AND PURPOSE

Small conductance calcium-activated potassium ($\text{K}_{\text{Ca}2.x}$) channels have a widely accepted canonical function in regulating cellular excitability. In this study, we address a potential non-canonical function of $\text{K}_{\text{Ca}2.x}$ channels in breast cancer cell survival, using *in vitro* models.

EXPERIMENTAL APPROACH

The expression of all $\text{K}_{\text{Ca}2.x}$ channel isoforms was initially probed using RT-PCR, Western blotting and microarray analysis in five widely studied breast cancer cell lines. In order to assess the effect of pharmacological blockade and siRNA-mediated knockdown of $\text{K}_{\text{Ca}2.x}$ channels on these cell lines, we utilized MTS proliferation assays and also followed the corresponding expression of apoptotic markers.

KEY RESULTS

All of the breast cancer cell lines, regardless of their lineage or endocrine responsiveness, were highly sensitive to $\text{K}_{\text{Ca}2.x}$ channel blockade. UCL1684 caused cytotoxicity, with LD_{50} values in the low nanomolar range, in all cell lines. The role of $\text{K}_{\text{Ca}2.x}$ channels was confirmed using pharmacological inhibition and siRNA-mediated knockdown. This reduced cell viability and also reduced expression of Bcl-2 but increased expression of active caspase-7 and caspase-9. Complementary to these results, a variety of cell lines can be protected from apoptosis induced by staurosporine using the $\text{K}_{\text{Ca}2.x}$ channel activator CyPPA.

CONCLUSIONS AND IMPLICATIONS

In addition to a well-established role for $\text{K}_{\text{Ca}2.x}$ channels in migration, blockade of these channels was potently cytotoxic in breast cancer cell lines, pointing to modulation of $\text{K}_{\text{Ca}2.x}$ channels as a potential therapeutic approach to breast cancer.

Abbreviations

AHP, afterhyperpolarization; FasR, faslodex-resistant cell line; $\text{K}_{\text{Ca}2.x}$ /SK channels, small conductance Ca^{2+} -activated potassium channels; No-RT, No reverse transcriptase; SK2-L, *KCNN2* channel long isoform; SK2-S, *KCNN2* channel short isoform; TamR, tamoxifen-resistant cell line

Tables of Links

TARGETS	
Ion channels^a	Enzymes^c
K _{Ca} 2.1 channels	Caspase-7
K _{Ca} 2.2 channels	Caspase-8
K _{Ca} 2.3 channels	Caspase-9
K _{Ca} 3.1 channels	Other proteins^d
Nuclear hormone receptors^b	Bcl-2
ER, oestrogen receptors	

LIGANDS
CyPPA
Faslodex, fulvestrant
NS8593
Staurosporine
Tamoxifen
UCL1684

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (^a ^b ^c ^d Alexander *et al.*, 2013a, b, c, d).

Introduction

SK channels or small conductance calcium-activated K⁺ channels (K_{Ca}2.x) are a family of K⁺ channels gated exclusively in response to rises in intracellular Ca²⁺ (Kohler *et al.*, 1996; Adelman *et al.*, 2012). The family consists of three channels (K_{Ca}2.1, K_{Ca}2.2, K_{Ca}2.3) with the pore forming subunit being encoded by the genes *KCNN1–KCNN3* and a closely related channel *KCNN4* also known as the intermediate conductance Ca²⁺-activated K⁺ channel (SK4, IK and K_{Ca}3.1). The SK (K_{Ca}2.x) channels do not have inherent Ca²⁺ binding domains and instead utilize calmodulin as their high affinity Ca²⁺ sensor (Xia *et al.*, 1998; Maylie *et al.*, 2004; Li *et al.*, 2009; Halling *et al.*, 2014). The Ca²⁺ sensitivity of the channel is then tightly regulated by the activity of a bound kinase (CK2) and phosphatase (PP2A). The canonical role of K_{Ca}2.x channels in physiology is in regulating cellular excitability, and to this end, these channels are widely expressed throughout the CNS where they play fundamental roles in generation of afterhyperpolarizations (AHPs), synaptic transmission and potentially in the generation of synaptic plasticity (Bond *et al.*, 2004; Faber *et al.*, 2005; Hammond *et al.*, 2006; Faber *et al.*, 2008; Nanou *et al.*, 2013). In addition to these canonical roles it has become evident that K_{Ca}2.x channels, much like other ion channels, have numerous non-canonical functions that span both excitable and non-excitable cells (Pardo *et al.*, 1998; Henney *et al.*, 2009; Huang *et al.*, 2012; Pardo and Stuhmer, 2014; Urrego *et al.*, 2014). Importantly, many ion channels have been implicated in processes related to cancer cell survival, motility, development and progression (Pedersen and Stock, 2013; Andersen *et al.*, 2014; Pardo and Stuhmer, 2014); in particular, expression of K_{Ca}2.3 channels has been conclusively linked to breast cancer cell motility and hence to metastases (Potier *et al.*, 2006; Potier *et al.*, 2010; Girault *et al.*, 2012; Chantome *et al.*, 2013). Here, we explore the role of K_{Ca}2.x channels in five widely used breast cancer cell lines, reflective of lineage and also endocrine resistance (Holliday and Speirs, 2011), using pharmacological modulators of K_{Ca}2.x channels to dissect the role of these channels in cell survival. Considering the huge global health

burden that breast cancer represents, where globally only lung cancer kills more women each year, it is desirable to open as many potential therapeutic research avenues as possible.

Here, we have shown that K_{Ca}2.x channels are expressed in four breast cancer cell models comprising estrogen receptor positive (ER+), endocrine responsive luminal A and B lines MCF-7 and BT-474 respectively and MCF-7-derived endocrine-resistant models, tamoxifen-resistant cell line (TamR) and faslodex-resistant cell line (FasR). Further, K_{Ca}2.3 channels were expressed in the commonly used estrogen receptor negative (ER[−]) breast cancer cell line, MDA-MB-231 cells. Pharmacological blockade using low MW inhibitors and siRNA demonstrated that K_{Ca}2.x channel knockdown was cytotoxic in not only breast cancer cell lines of varying ER expression but also two endocrine-resistant cell lines (TamR and FasR). Knockdown of K_{Ca}2.2 and K_{Ca}2.3 channels was accompanied by a considerable down-regulation of the anti-apoptotic protein Bcl-2 and a corresponding rise in the active cleaved form of caspase-7, one of the three main effector caspases and caspase-9. Thus, we suggest that K_{Ca}2.x channel blockade provided a pro-apoptotic stimulus, likely to activate the intrinsic pathway. This was complemented by our data illustrating that activation of K_{Ca}2.x channels markedly inhibited the effects of an apoptosis inducer (staurosporine) in a number of CNS-derived cell lines where the function of K_{Ca}2.x channels is better understood. Thus, we suggest a paradigm where aberrant activation of K_{Ca}2.x channels staves off cell death in breast cancer cell lines. These results clearly not only illustrate and compound the therapeutic potential of the blockade of K_{Ca}2.2 and K_{Ca}2.3 channels in primary and endocrine-resistant breast cancer, but also the importance of K_{Ca}2.x channels in cellular life and death decisions.

Methods

Cell lines

Breast cancer cells were provided by the Breast Cancer Molecular Pharmacology group at the School of Pharmacy and

Pharmaceutical Sciences, Cardiff University, UK. MCF-10a normal mammary cells were cultured in DMEM/F-12 (11320-074, Life Technologies) supplemented with 5% Horse serum, $10 \mu\text{g}\cdot\text{mL}^{-1}$ insulin (I9278, Sigma), $2 \mu\text{g}\cdot\text{mL}^{-1}$ epidermal growth factor (E9644, Sigma) and $0.5 \mu\text{g}\cdot\text{mL}^{-1}$ hydrocortisone (H0888, Sigma). MCF-7 cells were cultured in RPMI medium (Phenol Red⁺) supplemented with 5% FBS. TamR and FasR cells were previously derived by the Breast Cancer Molecular Pharmacology group from wild-type MCF-7 cells continually exposed to 100 nM 4-hydroxytamoxifen and Fulvestrant through to acquisition of stable resistance after 18 and 27 months respectively. These cells were maintained in RPMI medium (Phenol Red⁻) with 5% charcoal-stripped FBS. BT-474 cells were grown in DMEM with 10% FBS. MDA-MB-231 cells were grown in DMEM in a ratio 1:1 with F12 nutrient mixture with 10% FBS. All media was supplemented with 2 mM L-glutamine, $100 \text{ U}\cdot\text{mL}^{-1}$ penicillin and $100 \mu\text{g}\cdot\text{mL}^{-1}$ streptomycin, with cells being maintained at 37°C with 5% CO_2 .

RT-PCR

RT-PCR was carried out to determine the expression of $\text{K}_{\text{Ca}}2.x$ and $\text{K}_{\text{Ca}}3.x$ channels at the mRNA level. Negative controls represented RT-PCR reactions without RT and are denoted as 'No RT' throughout. Primers used are shown in Table 1.

Western blots

Western blotting was carried out to determine expression of $\text{K}_{\text{Ca}}2.2\text{-L}$, $\text{K}_{\text{Ca}}2.2\text{-S}$, $\text{K}_{\text{Ca}}2.3$, Bcl-2, full length and cleaved caspase-7 and cleaved caspase-8 and caspase-9. Breast cancer cell lines either untransfected or transfected with control siRNA or siRNA related to $\text{K}_{\text{Ca}}2.2$, 2.3 , were lysed, denatured and protein quantified using the BCA assay. Equal quantities of protein were loaded and separated by electrophoresis on 10% (w/v) SDS-PAGE gels at 120 V for 90 min . Proteins were wet blotted on to methanol-activated Hybond PVDF (GE healthcare, UK) membranes in ice-cold transfer buffer at 100 V for 60 min , blocked in 5% (w/v) dried skimmed milk in TBS-Tween[®] 20 for 3 h at room temperature and probed with rabbit polyclonal anti- $\text{K}_{\text{Ca}}2.2$ (long isoform) (1:1000) (Abcam), rabbit polyclonal anti- $\text{K}_{\text{Ca}}2.2$ (short isoform) (1:1000) (Abcam), rabbit polyclonal anti- $\text{K}_{\text{Ca}}2.3$ (1:1000) (Abcam), rabbit monoclonal anti-Bcl-2 (1:1000) (Abcam), rabbit monoclonal anti-caspase-7 full length and cleaved (1:1000) (Abcam), rabbit anti-cleaved caspase-7 (1:1000) (Cell Signalling Technology), mouse anti-GAPDH (1:50 000) (Sigma-Aldrich) rabbit polyclonal anti- $\text{K}_{\text{Ca}}2.1$ (1:1000) (Abcam), rabbit monoclonal anti-GAP-43 (1:1000) (Abcam), cleaved caspase-8 (1:1000) (Cell Signalling Technology), cleaved caspase-9 (1:1000) (Cell Signalling Technology) all applied in 5% (w/v) dried skimmed milk and TBS-Tween 20[®] mixture and incubated at 4°C overnight. The next day,

Table 1

Primer sequences for all K_{Ca} channel targets in human and murine cell lines.

Genes		Primers	Amplicon (bp)
Human β -actin	Forward	5'-CCCAGCCATGTACGTTGCTA-3'	126
	Reverse	5'-AGGGCATACCCCTCGTAGATG-3'	
Human KCNN1	Forward	5'-TGGACACTCAGCTACCAAG-3'	208
	Reverse	5'-TTAGCCTGGTCGTTTCAGCTT-3'	
Human KCNN2	Forward	5'-CAAGCAAACACTTTGGTGA-3'	249
	Reverse	5'-CCGCTCAGCATTGTAAGTGA-3'	
Human KCNN3	Forward	5'-TCCATTGGTTATGGGGACAT-3'	180
	Reverse	5'-CTTGGTGAGCTGAGTGTCCA-3'	
Human KCNN4	Forward	5'-GAGAGGACAGGCTGTTATTGC-3'	215
	Reverse	5'-ACGTGCTTCTCTGCCTTGTT-3'	
Mouse β -actin	Forward	5'-TGTTACCAACTGGGACGACA-3'	165
	Reverse	5'-GGGGTGTGAAGGTCTCAA-3'	
Mouse KCNN1	Forward	5'-GAAGCTTGGGTGAACTGAGC-3'	232
	Reverse	5'-CCATTAAGGAATCCCCAGGT-3'	
Mouse KCNN2	Forward	5'-TCTGATTGCCAGAGTCATGC-3'	250
	Reverse	5'-CCACATTGCTCCAAGGAAGT-3'	
Mouse KCNN3	Forward	5'-ACTTCAACACCCGATTCTGTC-3'	191
	Reverse	5'-GGAAAGGAACGTGATGGAGA-3'	
Mouse KCNN4	Forward	5'-AAGCACACTCGAAGGAAGGA-3'	215
	Reverse	5'-CCGTCGATTCTCTTCTCCAG-3'	

membranes were washed and incubated with the relevant secondary antibody diluted in 5% (w/v) dried skimmed milk TBS-Tween 20®, for 60 min at room temperature: Anti-rabbit IgG (1:10 000) (Cell Signalling Technology: no. 7074). After a series of washes, proteins were detected using the ECL Plus Western blotting chemiluminescent detection system (Pierce, UK), and signals were captured on Hyperfilm ECL autoradiography film (GE healthcare, UK) in a dark room. The exposure times for the detection were the following: K_{Ca}2.2-L, K_{Ca}2.2-S, K_{Ca}2.3, Bcl-2, GAP43, full length and cleaved caspase-7, cleaved caspase-8, cleaved caspase-9 for 2 min, 3 min, 2 min, 2 min, 3–30 min, 30 s, 3 min and 3 min respectively and 1 min for GAPDH for each blot after stripping and reprobing. Western blot results were semi-quantified using ImageJ software following, as closely as possible, protocols set out by Gassmann *et al.*, 2009 (Gassmann *et al.*, 2009). Firstly, images were converted to 8-bit grayscale (256 gray shades), and rectangles were drawn (30% of the lane width – tight as possible with respect to band height) around each band and lanes were plotted. As pointed out in Gassmann *et al.* (2009), background correction can actually impair the densitometry values obtained when using ImageJ software so none was undertaken in this study (Gassmann *et al.*, 2009). The peak heights of relevant bands were utilized and compared with loading control (GAPDH). ImageJ enables each peak to be labeled with its size, and then it can be expressed as a percentage of the total size of all of the highlighted peaks on the blot. Using the relative densities of the loading controls, adjusted density values for protein of interest were calculated, by dividing the density of each sample by the loading control.

Cell viability assays

Cells were seeded in triplicate into 96-well plates at a density of 8×10^3 cells per well. The cells were then exposed to treatment with SK channel modulators for 72 h, each day cells being refreshed with the relevant media for the individual cell line supplemented with the compound of interest. For the MTS assay, 40 mg MTS powder reagent (Promega, UK) was dissolved in 20 mL PBS. This mixture (2.5 mL) was added to PMS (120 µL) to form an MTS–PMS mix at a ratio of 20:1. The MTS–PMS mixture (20 µL) was pipetted into each well (ratio, 1:5), protected from light, and incubated for 3 h. Results were read in a LT-5000MS ELISA plate reader where relative cell viability was measured as a function of absorbance.

siRNA transfection

siRNA was purchased from Eurofins Genomics.

GFP control siRNA 5'-GGCUACGUCCAGGAGCGCACC-3',

K_{Ca}2.2 siRNA 5'-GCAUUGGAGCACUUAUAA-3'(Exon 3)

K_{Ca}2.3 siRNA 5'-UUGUUGUUAUGGUGAUAGA-3'(Exon 1)

Cells were plated in 6-well plates seeded at a density of 20×10^4 cells per well and left for 24 h. siRNA (50 nM K_{Ca}2.2 siRNA in MCF-7 and BT-474 cells and 80 nM of K_{Ca}2.3 siRNA in MDA-MB-231 cells) was incubated with Oligofectamine™ (Invitrogen Life Technologies) at room temperature in OptiMEM for 15 min. Cells were then incubated for 72 h. Transfected cells were harvested from 6-well plates and

reseeded in 96-well plates for 72 h, each day cells being refreshed with normal medium. Cell viability was then determined using an MTS assay and protein expression of the relevant K_{Ca}2.x channel, Bcl-2, caspase-7, caspase-8 and caspase-9 was quantified.

Microarray

A quantitative approach supplementary to PCR was employed to explore relative expression of transcripts encoding SK1-4 channels. The technique involves labelling of an RNA sample and allowing hybridization with gene-specific targets on an array (Affymetrix 1.0ST GeneChip platform). RNA was extracted by the Breast Cancer Molecular Pharmacology group from triplicate preparations of three different cell lines (MCF-7, TamR and FasR cells) at three passage numbers. Array analysis was then carried out using the Affymetrix GeneChips and the resultant expression data for the SK channels of interest subsequently analysed using commercial GENESIFTER software, considering heatmap profile and expression level (log₂intensity).

Data analysis

For cell viability assays, data are represented as a mean of each group \pm SEM normalized to control. GraphPad Prism 6 was used to analyze the data using either Student's *t*-test (densitometry data), one-way ANOVA or two-way ANOVA, with either Dunnett's or Tukey's post hoc tests employed, not including controls of normalized data. Data were considered statistically significant when $P < 0.05$. If a more stringent statistical threshold is set, it is noted in the figure legend. 95% confidence intervals are displayed where appropriate.

Materials

Pharmacological modulators of K_{Ca}2.x and K_{Ca}3.x channels were purchased from Tocris Bioscience; CyPPA (Cat. no. 2953), UCL1684 (Cat. no. 1310) and NS6180 (Cat. no. 4864). NS8593 (Cat. no. N2538) was purchased from Sigma-Aldrich, UK.

Results

K_{Ca}2.2, 2.3 channel expression and modulation in breast cancer cell lines

First, we looked at the expression of all three K_{Ca}2.x and K_{Ca}3.1 channels in three widely used breast cancer cell lines of varying lineage, namely, MCF-7 (Luminal A: ER⁺, HER2⁻), BT-474 (Luminal B: ER⁺, HER2⁺) and MDA-MB-231 cells (Claudin-low: ER⁻, HER2⁺) (Holliday and Speirs, 2011). In the adenocarcinoma MCF-7 cells, K_{Ca}2.2 and K_{Ca}3.1 channels were both present at the mRNA level (Figure 1A), and K_{Ca}2.2 channel expression was confirmed at the protein level using Western blotting (Figure 1B and Supporting Information Fig. S1). In fact two isoforms of the K_{Ca}2.2 channel were identified which are known to form heteromultimeric channels; isoform a, which is a longer

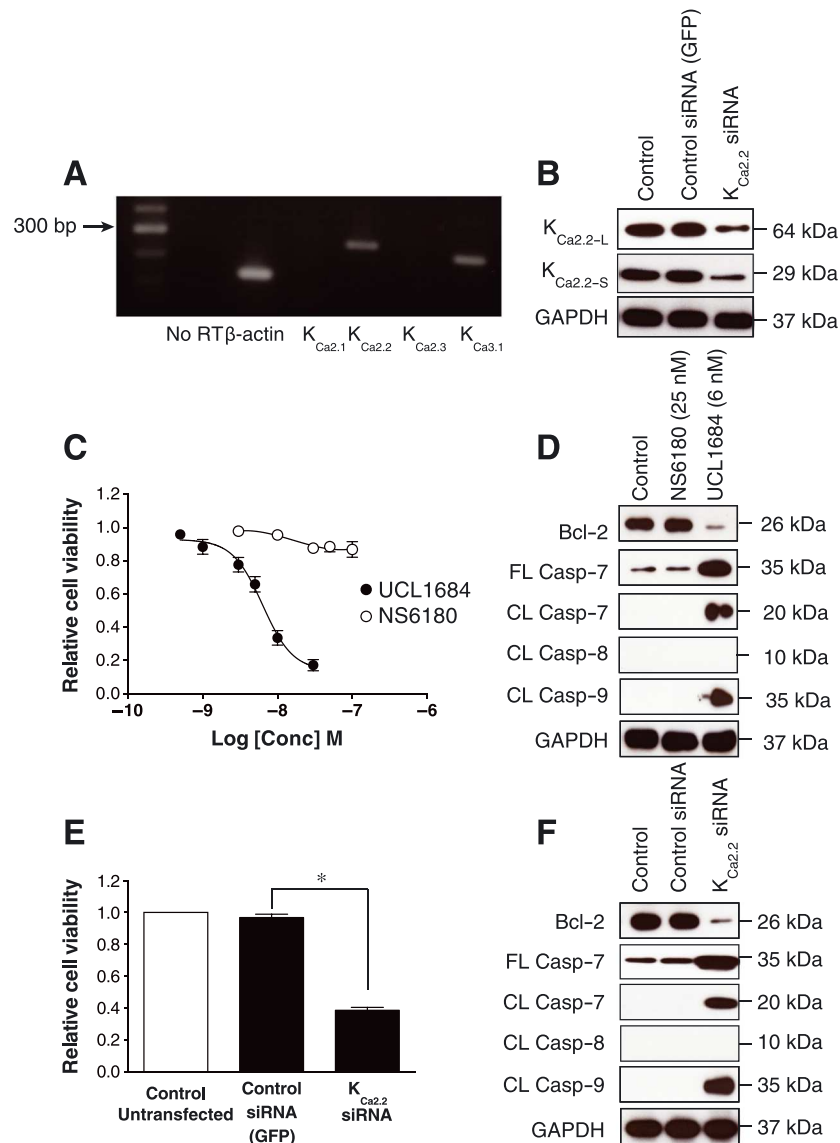


Figure 1

Expression and pharmacological/siRNA-mediated modulation of K_{Ca2.2} channels in MCF-7 cells. (A) RT-PCR illustrates the presence of K_{Ca2.2} (249 bp) and K_{Ca3.1} channels (215 bp). Negative control represents RT-PCR reactions without RT (No RT), and positive control is β -actin (126 bp). (B) Western blot analysis of K_{Ca2.2} expression demonstrating knockdown of both K_{Ca2.2} long (L) and short (S) isoforms by siRNA and no effect of control siRNA (GFP targeted). Relative expression to GAPDH was calculated using densitometry (Supporting Information Fig. S1). (C) Dose-response curve illustrating the effect of UCL1684 (K_{Ca2.x}) and NS6180 (K_{Ca3.1}) on MCF-7 viability after treatment for 72 h (data points represent mean \pm SEM; $n = 9$). (D) Effect of pharmacological blockers UCL1684 (K_{Ca2.x}) and NS6180 (K_{Ca3.1}) on the expression of Bcl-2, full length (FL) and cleaved (CL) form of caspase-7 and cleaved caspase-8 and caspase-9. (E) Effect of siRNA-mediated knockdown of K_{Ca2.2} channels on MCF-7 viability. * $P < 0.01$; significantly different as indicated; $n = 9$. (F) Expression of Bcl-2 and full length (FL) and cleaved (CL) form of caspase-7, caspase-8 and caspase-9 were determined after 72 h of exposure to K_{Ca2.2} channel siRNA.

isoform with a large N-terminal extension (SK2-L) and isoform b, a shorter variant (SK2-S) (Allen *et al.*, 2011).

We then looked at whether MCF-7 cells were sensitive to K_{Ca2.x} and K_{Ca3.1} channel modulators (Figure 1C and Supporting Information Fig. S2). While MCF-7 cells were relatively insensitive to the K_{Ca3.1} channel blocker NS6180 (LD₅₀ \gg 0.1 μ M), they were highly sensitive to the K_{Ca2.x} channel blocker UCL1684 with an LD₅₀ of 6.3 nM (4.9–8.1; 95% confidence interval, $n = 9$) (Figure 1C). In contrast, the K_{Ca2} channel activator CyPPA (5–30 μ M) (Hougaard *et al.*, 2007) increased cell

viability by up to ~60% (Supporting Information Fig. S2). This effect was accompanied by an increase in Bcl-2 levels and could be completely abolished by the addition of UCL1684 to block K_{Ca2.x} channels (Figure S2).

After pharmacological inhibition of K_{Ca2.2} channels using UCL1684, we observed a marked reduction in Bcl-2 expression (approx. 10-fold) and a corresponding rise in the expression of caspase-7 (approx. fivefold), one of the three main effector caspases (Figure 1D and Supporting Information Fig. S3). It is important to note that the caspase-7

antibody used here binds to both full length and activated caspase-7 post-enzymic cleavage. In the control, the activated caspase-7, as we would expect, was absent, but on treatment with the $K_{Ca2.2}$ channel blocker, both full length and most importantly cleaved caspase-7 was increased (Figure 1D). To further probe the potential mechanism of UCL1684, we looked at cleaved caspase-8 (extrinsic apoptotic pathway) and caspase-9 (intrinsic apoptotic pathway). We did not observe an increase in caspase-8 expression, but there was a marked rise in caspase-9 levels (Figure 1D).

Interestingly, the effects of UCL1684 were mirrored using siRNA-mediated knockdown of $K_{Ca2.2}$ channels in MCF-7 cells, confirmed using Western blotting (Figure 1B, E and Supporting Information Fig. S1). We found that the siRNA used was as potent at knocking down the SK2-L

(long) isoform as the SK2-S (short) isoform. Under these conditions, there was again a vast reduction in Bcl-2 expression (up to 25-fold) and a corresponding rise in the expression of caspase-7 and caspase-9 (Figure 1F and Supporting Information Fig. S1).

$K_{Ca2.2}$ channel expression was also confirmed in another luminal endocrine responsive cell line BT-474 (Figure 2A and B). Again, these cells were very sensitive to UCL1684 with an LD_{50} of 4.0 nM (3.1–5.1; 95% confidence interval, $n = 9$) (Figure 2C). This treatment again seemed to activate the intrinsic pathway resulting in a rise in caspase-9 expression (Figure 2D). These effects could be mimicked by siRNA-mediated knockdown of $K_{Ca2.2}$ channels (Figure 2B and E). The knockdown mediated by siRNA was accompanied by a marked decrease in Bcl-2 and an up-regulation of caspase-7

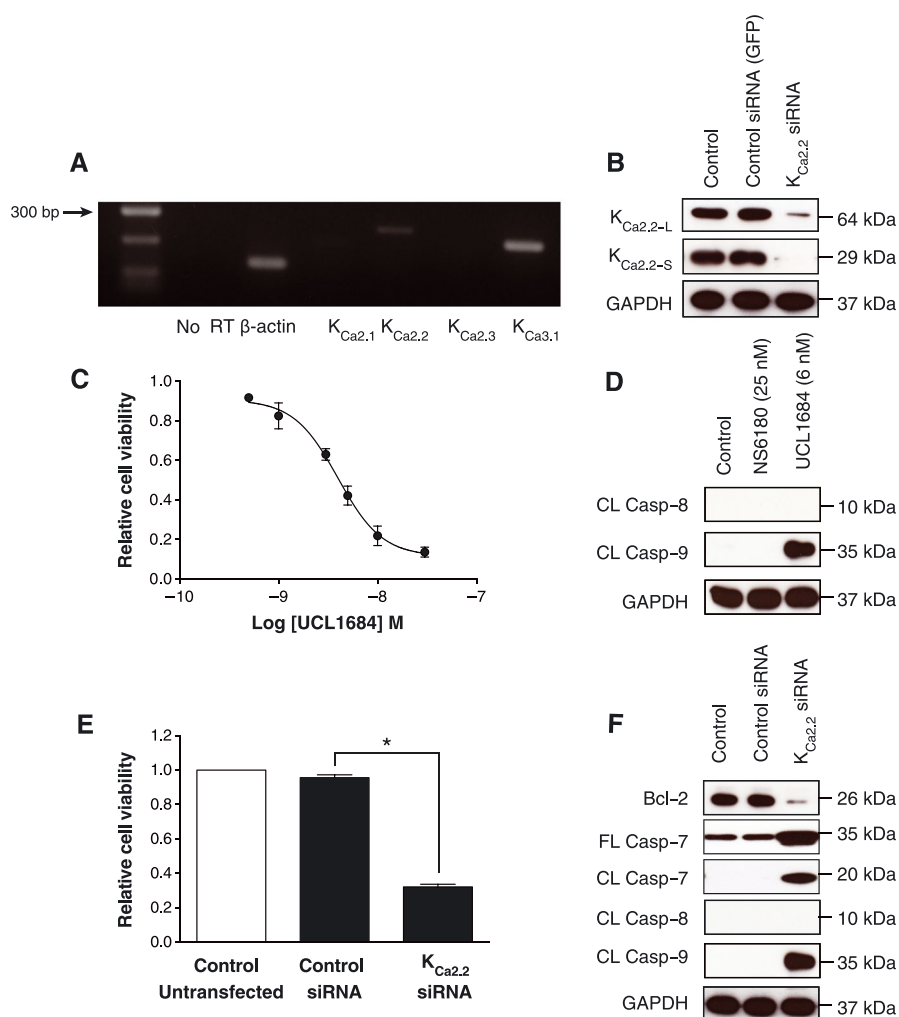


Figure 2

Expression and pharmacological/siRNA-mediated modulation of $K_{Ca2.2}$ channels in BT-474 cells. (A) RT-PCR illustrates the presence of $K_{Ca2.2}$ (249 bp) and $K_{Ca3.1}$ channels (215 bp). (B) Western blot analysis of $K_{Ca2.2}$ channel expression demonstrating knockdown of both $K_{Ca2.2}$ long (L) and short (S) isoforms by siRNA and no effect of control siRNA. Relative expression compared with GAPDH was calculated using densitometry (Supporting Information Fig. S1). (C) Dose-response curve illustrating the effect of UCL1684 ($K_{Ca2.x}$) on BT-474 cell viability. Data points represent mean \pm SEM; $n = 9$. (D) Effect of pharmacological blockers UCL1684 ($K_{Ca2.x}$) and NS6180 ($K_{Ca3.1}$) on the expression of cleaved caspase-8 and caspase-9. (E) Effect of siRNA-mediated knockdown of $K_{Ca2.2}$ channels on BT-474 cell viability * $P < 0.01$; significantly different as indicated; $n = 9$. (F) Expression of Bcl-2, full length (FL) and cleaved (CL) form of caspase-7 and cleaved caspase-8 and caspase-9 after 72 h exposure to $K_{Ca2.2}$ channel siRNA.

and caspase-9, identical to the effects seen in MCF-7 cells (Figure 2F and Supporting Information Fig. S1).

In contrast, the claudin-low cell line MDA-MB-231 that is ER⁻ and HER⁻ seemed not to express K_{Ca}2.2 channels but did express K_{Ca}2.3 channels (Figure 3A and B). UCL1684 is slightly more selective for K_{Ca}2.1 and K_{Ca}2.2 channels so, in addition, we tested NS8593 (another generic K_{Ca}2.x channel blocker) on the growth of MDA-MB-231 cells. Both demonstrated potent cytotoxicity with LD₅₀ values of 6.1 nM (4.8–7.7; 95% confidence interval, *n* = 9) and 813 nM (626–1057; 95% confidence interval, *n* = 9) respectively over the time frame of the experiments (Figure 3C and D). Furthermore, as in both the luminal cell lines expressing K_{Ca}2.2 channels, siRNA-mediated knockdown of K_{Ca}2.3 channels had a profound cytotoxic effect and caused a decrease in Bcl-2 expression and an increase in cleaved caspase-7 and caspase-9 expression (Figure 3E and F and Supporting Information Fig. S1).

After seeing these potent effects of K_{Ca}2 channel inhibition on breast cancer cell lines, we looked at whether the

same could be seen in the non-cancerous mammary epithelial cell line MCF-10A. We probed expression at the mRNA level and the protein level, and no K_{Ca}2.x channel isoforms could be detected (Figure 4A and B). Thus, unsurprisingly, neither treatment with UCL1684 nor siRNA specific for K_{Ca}2.2 or K_{Ca}2.3 channels had any effect on MCF-10A cell viability (Figure 4C and D).

Effect of K_{Ca}2.x channel blockers on endocrine-resistant breast cancer cell lines

Next, we sought to decipher whether K_{Ca}2.x channels were expressed and whether their blockade was as potently cytotoxic in two endocrine-resistant cell lines. TamR and FasR cell lines were previously derived from MCF-7 cells which have been treated continuously until they acquired resistance to the endocrine agents, tamoxifen and faslodex respectively, two widely used treatments for breast cancer (Knowlden *et al.*, 2003; Knowlden *et al.*, 2005; Nicholson *et al.*, 2005).

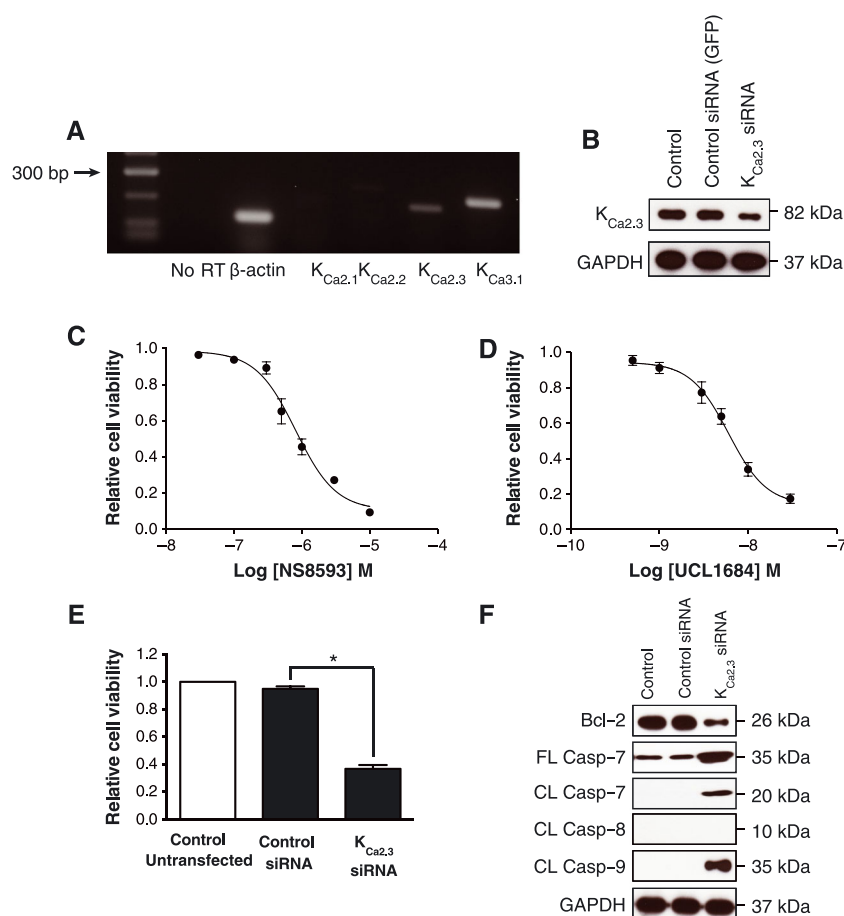


Figure 3

Expression and pharmacological/siRNA-mediated modulation of K_{Ca}2.3 channels in MDA-MB-231 cells. (A) RT-PCR illustrates the presence of K_{Ca}2.3 (180 bp) and K_{Ca}3.1 channels (215 bp). (B) Western blot analysis of K_{Ca}2.3 channel expression demonstrating knockdown by siRNA and no effect of control siRNA. Relative expression was calculated using densitometry (Supporting Information Fig. S1). Dose-response curve illustrating the effect of (C) NS8593 and (D) UCL1684 on MDA-MB-231 cell viability. Data shown are means ± SEM; *n* = 9. (E) Effect of siRNA-mediated knockdown of K_{Ca}2.3 channels on MDA-MB-231 cell viability. * *P* < 0.05; significantly different as indicated; *n* = 9. (F) Expression of Bcl-2, full length (FL) and cleaved (CL) form of caspase-7 and cleaved caspase-8 and caspase-9 in response to 72 h exposure to K_{Ca}2.3 channel siRNA.

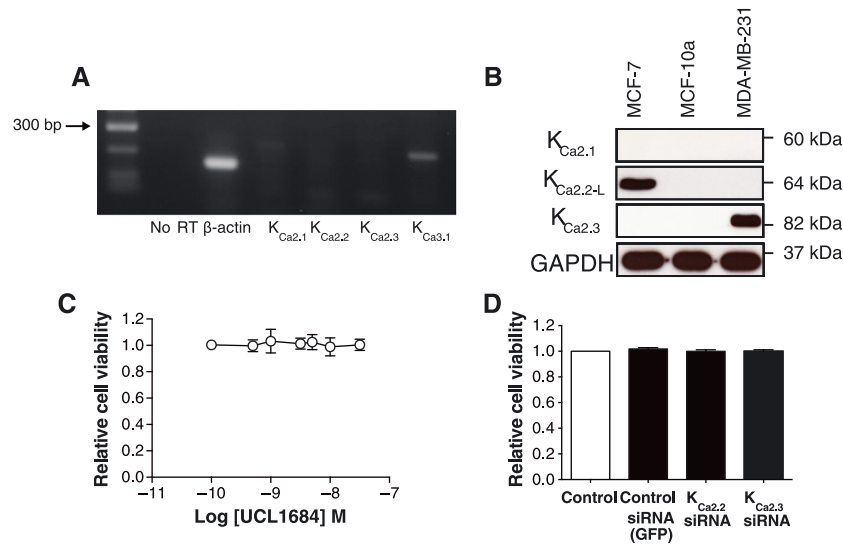


Figure 4

Expression and pharmacological/siRNA-mediated modulation of $K_{Ca2.x}$ channels in the human mammary epithelial cell line MCF-10A. (A) RT-PCR illustrates a faint band for $K_{Ca2.1}$ (208 bp) and $K_{Ca3.1}$ channels (215 bp). Negative control represents RT-PCR reactions minus RT (No RT) and positive control is β -actin (126 bp). (B) Western blot analysis of $K_{Ca2.x}$ channel expression demonstrates that although a faint PCR band is present relating to the mRNA signal for $K_{Ca2.1}$ channels, they were not expressed at the protein level (comparison with MCF-7 and MDA-MB-231 cell lines are shown). (C) Dose-response curve illustrating the effect of UCL1684 ($K_{Ca2.x}$) on MCF-10A viability after treatment for 72 h. Data shown are means \pm SEM; $n = 9$. (D) Effect of siRNA specific to $K_{Ca2.2}$ and $K_{Ca2.3}$ channels on cell viability of MCF-10A cells ($n = 7$).

TamR and FasR *KCNN1–KCNN4* expression was probed using PCR and Western blotting (Figure 5A and B). Both FasR and TamR expressed $K_{Ca2.2}$ and $K_{Ca3.1}$ channels at the mRNA

level with expression of $K_{Ca2.2}$ channels being confirmed in both using Western blotting. We also undertook microarray analysis on relative expression of $K_{Ca2.x}$ and $K_{Ca3.x}$ channels

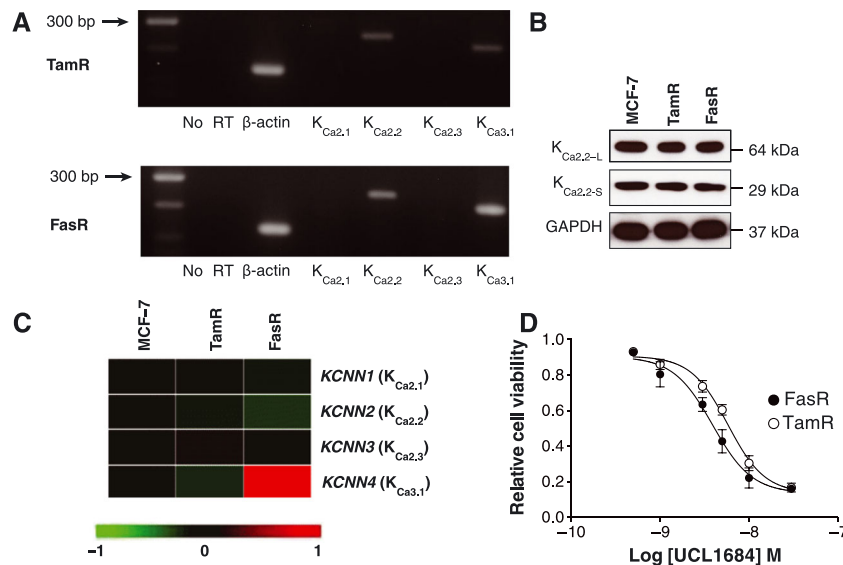


Figure 5

Expression and pharmacological modulation of $K_{Ca2.2}$ channels in the endocrine-resistant cell lines TamR and FasR. (A) RT-PCR illustrates the presence of $K_{Ca2.2}$ (249 bp) and $K_{Ca3.1}$ channels (215 bp) at the mRNA level in TamR cells (upper panel) and FasR cells (lower panel). (B) Western blot analysis of $K_{Ca2.2}$ channel expression at the protein level in MCF-7, TamR and FasR cells, looking at both $K_{Ca2.2-S}$ and the $K_{Ca2.2-L}$ isoforms. (C) Genesifter Microarray heatmap illustrating an up-regulation (red) of $K_{Ca3.1}$ channels in FasR cells compared with MCF-7 cells; $P < 0.01$. There was marginal down-regulation of $K_{Ca2.2}$ channels in FasR and $K_{Ca3.1}$ in TamR cells; $P > 0.05$. (D) Dose-response curve illustrating the effect of UCL1684 on TamR and FasR cell lines. Data shown are means \pm SEM; $n = 9$.

in TamR and FasR cell lines comparing them with the expression seen in the parental cell line, MCF-7 (Figure 5C). This analysis confirmed the expression of $K_{Ca2.2}$ and $K_{Ca3.1}$ channels in MCF-7 and the resistant models (KCNN2 and KCNN4 respectively; Genesifter \log_2 intensity >7), with no expression of either KCNN1 or KCNN3. There was a marked increase (fourfold) in the expression of $K_{Ca3.1}$ channels in FasR compared with MCF-7 cells apparent on the heatmaps (P -value < 0.01), but no significant change in mRNA expression was seen for any $K_{Ca2.x}$ isoform versus MCF-7 (Figure 5C). Both resistant cell lines exhibited high sensitivity to the $K_{Ca2.x}$ channel blocker UCL1684 with an LD_{50} of 3.9 nM (2.9–5.2; 95% confidence interval, $n = 9$) for FasR cells and 5.8 nM (4.8–6.9; 95% confidence interval, $n = 9$) for TamR cells (Figure 5D).

Are $K_{Ca2.x}$ channel blockers generally cytotoxic?

As a comparison, we tested the $K_{Ca2.x}$ channel blockers on other cell lines, both expressing and not expressing $K_{Ca2.x}$ channels, in order to determine how specific these observed effects on breast cancer lines might be. Here, we used CNS-derived cell lines as the functional role of $K_{Ca2.x}$ channels in these cell types *in vivo* have been widely probed. Again, we probed $K_{Ca2.x}$ channel expression at the mRNA level and protein level and confirmed the presence of a neuronal marker in differentiated SH-SY5Y cells, namely, GAP-43 (Figure 6 and Supporting Information Fig. S4). Neither UCL1684 (10 nM) nor NS8593 (1 μ M) affected the cell viability of SH-SY5Y (neuronal – $K_{Ca2.1}$ and $K_{Ca2.3}$) cells or MOG-G-UVW (astrocytic – $K_{Ca3.1}$) cells (Figure 6A–C and Supporting Information Fig. S4). They were also without effect on a murine striatal cell line *STHdh*, which is the control

line for a murine model of Huntington's disease (Trettel *et al.*, 2000). These cells were chosen as striatal cells from the wild-type, heterozygous and homozygous mice in this particular model display differential expression of $K_{Ca2.x}$ channels, which in itself is noteworthy (Figure 6D and E and Supporting Information Fig. S4). Of specific relevance is that in the striatal neurons of homozygous mice (*STHdh*^{Q111/Q111}) displaying the Huntington-like phenotype, we could only identify $K_{Ca2.1}$ (SK1) channels, which cannot form functional homomeric channels in mouse or rat (Benton *et al.*, 2003; Adelman *et al.*, 2012). This means homozygous mice showing the Huntington's phenotype would most likely be without AHP currents driven by $K_{Ca2.x}$ channels releasing the 'braking influence' on neuronal excitability that they confer. In fact, this is functionally what is seen in another mouse model presenting with a Huntington-like phenotype (Moriguchi *et al.*, 2006). In this model, the expression of $K_{Ca2.x}$ channels is not lost, but an apamin-sensitive AHP current is functionally lost. This initial expression analysis is the subject of an ongoing study. Although this is obviously not an exhaustive cytotoxicity screen, it does illustrate some specificity of the effects of UCL1684 and NS8593 on cancer cells.

Activation of $K_{Ca2.x}$ channels ameliorates cell death induced by staurosporine

Staurosporine is a natural product isolated from *Streptomyces staurosporeus*, which is widely used to induce apoptosis (Nakagawa *et al.*, 2000; Belmokhtar *et al.*, 2001; Dong *et al.*, 2001; Marti *et al.*, 2006; Sullivan *et al.*, 2008). So we used CNS-derived cell lines where cell proliferation was insensitive to $K_{Ca2.x}$ channel blockade to probe the question, can activation of these channels ameliorate the cellular response to an apoptotic stimulus? To this end, we treated three cell lines

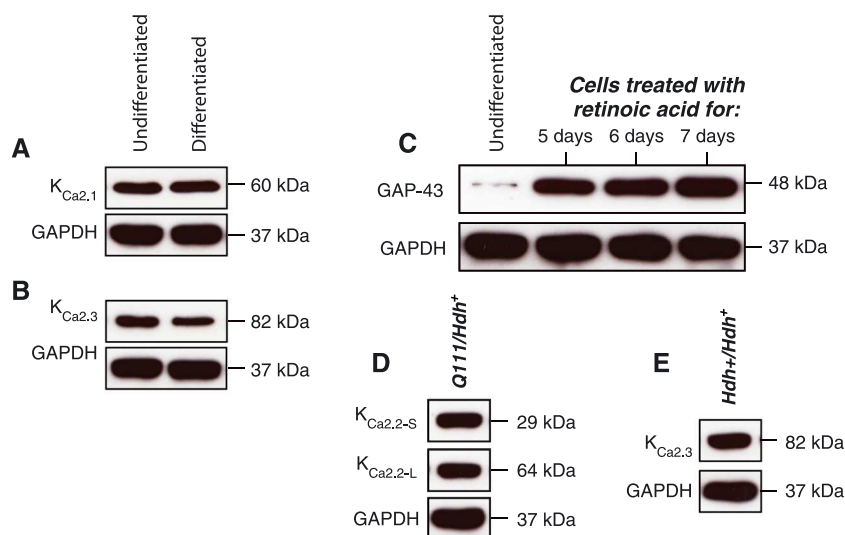


Figure 6

Expression of $K_{Ca2.x}$ in CNS-derived cell lines. (A) Expression of $K_{Ca2.1}$ channels in differentiated and undifferentiated SH-SY5Y. (B) Expression of $K_{Ca2.3}$ channels in differentiated and undifferentiated SH-SY5Y. (C) Confirmation that SH-SY5Y cells are differentiated in response to retinoic acid treatment using GAP-43 as a neuronal marker. (D) Expression of $K_{Ca2.2}$ channels, both long and short isoforms, in heterozygous mutant murine striatal neurons (*STHdh*^{Q111/Hdh+}). (E) $K_{Ca2.3}$ channel expression in wild-type murine striatal neurons *STHdh*.

over 24 h with staurosporine (30–100 nM) and co-treated them with the $K_{Ca2.x}$ channel activator CyPPA. The response of these cell lines to staurosporine alone is consistent with previous reports (LD_{50} ~10–100 nM for 24 h treatment) (Sullivan *et al.*, 2008; Sodja *et al.*, 2014). The co-treatment with CyPPA then increased cell viability by up to 30% when compared with staurosporine alone (Figure 7A–C). This clearly illustrates that in addition to $K_{Ca2.x}$ channel knockdown providing a pro-apoptotic signal in breast cancer cell lines, activation of these channels can ameliorate the effects of an apoptotic stimulus. To ensure these effects were not simply due to an increase in viability as we observed over a 72 h period, we tested whether CyPPA alone affected cell viability over 24 h. CyPPA (50 μ M) did not affect cell viability on any of the CNS-derived cell lines (Figure 7A–C). Furthermore, we showed that the effects of CyPPA on staurosporine-induced cell death was abolished by the application of the $K_{Ca2.x}$ channel blocker UCL1684 at a dose comparable with the LD_{50} doses in cancer cells (Figure 7D and E).

Discussion

We examined here the cytotoxicity resulting from modulation of $K_{Ca2.x}$ channels in breast cancer cell lines. Previously, $K_{Ca2.x}$ channels have been implicated in cancer cell migration (Potier *et al.*, 2006; Potier *et al.*, 2010) and in line with other data, the expression profile of $K_{Ca2.2}$ and $K_{Ca2.3}$ channels demonstrated here mirrors the fact that MDA-MB-231 cells (expressing $K_{Ca2.3}$ channels) are more motile than MCF-7 cells, which lack these channels (Krueger *et al.*, 2001; Potier *et al.*, 2006; Potier *et al.*, 2010; Girault *et al.*, 2012). Our results clearly show that three widely used breast cancer cell lines that cumulatively reflect key breast cancer subtypes (luminal A, B, claudin low; ER^+ and ER^-) not only express $K_{Ca2.x}$ channels but also are exquisitely sensitive to their blockade, with the compound UCL1684 causing decreases in cell viability with LD_{50} values in the nanomolar range (~5 nM). These effects could be reproduced in each of these cell lines using siRNA-mediated knockdown of the relevant $K_{Ca2.x}$ channels. Despite a lack of complete siRNA-mediated knockdown of $K_{Ca2.x}$ channel expression in MCF-7 and MDA-MB-231 cells, substantial effects were still seen on cell viability. Furthermore, both siRNA-mediated knockdown and pharmacological blockade were accompanied by a fall in the anti-apoptotic protein Bcl-2 and a rise in the active form of the effector caspase-7 and caspase-9. From this, we suggest that $K_{Ca2.x}$ channel blockade is providing a pro-apoptotic signal in particular, activating the intrinsic pathway (caspase-9 is activated during the intrinsic pathway). To date, there is much evidence of a role for K^+ channels in proliferation and cell cycle progression (Huang *et al.*, 2012; Urrego *et al.*, 2014). Here, we assume that these breast cancer cells are 'high-jacking' $K_{Ca2.x}$ channels to drive growth by resisting apoptosis which is largely supported by the fact that the $K_{Ca2.x}$ channel activator CyPPA, which acts by increasing the channel sensitivity to intracellular Ca^{2+} , actually increased cell viability in both cell lines tested over 72 h with a corresponding rise in Bcl-2 levels (MCF-7 and MDA-MB-231) (Hougaard *et al.*, 2007). CyPPA has previously been shown not to affect cell viability in microglia, but this was only up to 48 h treatment, and we also show similar data

when we treat CNS-derived cells over a shorter time frame (Figure 7), that is, no increase in cell viability (Dolga *et al.*, 2012).

Previous reports have suggested that breast cancer cell line growth is not sensitive to apamin, another $K_{Ca2.x}$ channel blocker. So an obvious question is why the sensitivity to UCL1684 and NS8593 but not apamin? For example, Potier *et al.* (2006) showed that apamin (100 nM) did not affect viability in MDA-MB-435 cells (Potier *et al.*, 2006) over a 24 h period. However, in our experiments, we challenged cells over 72 h. In addition, $K_{Ca2.2}$ channel expression in melanoma cells is increased under hypoxic conditions, and it is only then that these cells become sensitive to apamin (Tajima *et al.*, 2006). So a lack of sensitivity of cell viability to apamin may also be related to relative expression levels. Finally, both UCL1684 and NS8593 are low MW inhibitors, whereas apamin is a peptide. Thus, the effects seen may be related to their relative membrane permeability and hence to $K_{Ca2.2}$ channel blockade at the mitochondrial level rather than at the plasma membrane. With this in mind, it is important to note that there is a burgeoning literature on the role of mitochondrial $K_{Ca2.x}$ channels in cell physiology (Dolga *et al.*, 2011; Dolga and Culmsee, 2012; Dolga *et al.*, 2013; Dolga *et al.*, 2014; Richter *et al.*, 2015). Mitochondrial membrane potential is linked to the intrinsic apoptotic pathway, and thus, modulation of $K_{Ca2.x}$ channels in mitochondria may explain the effects seen in the cancer cell lines.

Blockade of $K_{Ca2.x}$ channels not only reduced cell viability in the endocrine responsive luminal cell lines but also reduced viability in a dose-dependent manner in two endocrine-resistant models (TamR and FasR). The $K_{Ca2.x}$ channel blocker UCL1684 LD_{50} values again were in the low nanomolar range, with FasR cells being slightly more sensitive to UCL1684 than other cell lines. This is perhaps in part due to a slightly lower expression level of $K_{Ca2.2}$ channels, compared with both MCF-7 and TamR cells (although microarray data did not show a significant difference in mRNA levels).

Furthermore, when tested at two to four times the LD_{50} for breast cancer cell lines on five other cell lines of central nervous system origin, the same K_{Ca2} channel blockers were without effect. In addition, UCL1684 did not affect the non-cancerous mammary cell line MCF-10A. From this, we suggest that the cytotoxic effects seen in breast cancer cells are not widespread and may be suggestive of aberrant or specialized function of $K_{Ca2.x}$ channels in breast cancer cell lines. We went further using these cells, which were insensitive to $K_{Ca2.x}$ channel blockade. In reverse to what is seen in breast cancer lines, we wanted to know whether $K_{Ca2.x}$ channel activation could ameliorate the effects of an apoptosis inducer, staurosporine. Interestingly, and totally internally consistent with our results, CyPPA treatment prevented the reduction in viability in three cell lines of CNS origin caused by staurosporine. The protection afforded by CyPPA was abolished by the addition of a $K_{Ca2.2}$ channel blocker. All of which clearly indicated a role of $K_{Ca2.x}$ channels in cellular life and death decisions.

In conclusion, we have clearly demonstrated the cytotoxicity of $K_{Ca2.x}$ channel knockdown in breast cancer cell lines. The marked sensitivity of these cell lines, spanning breast

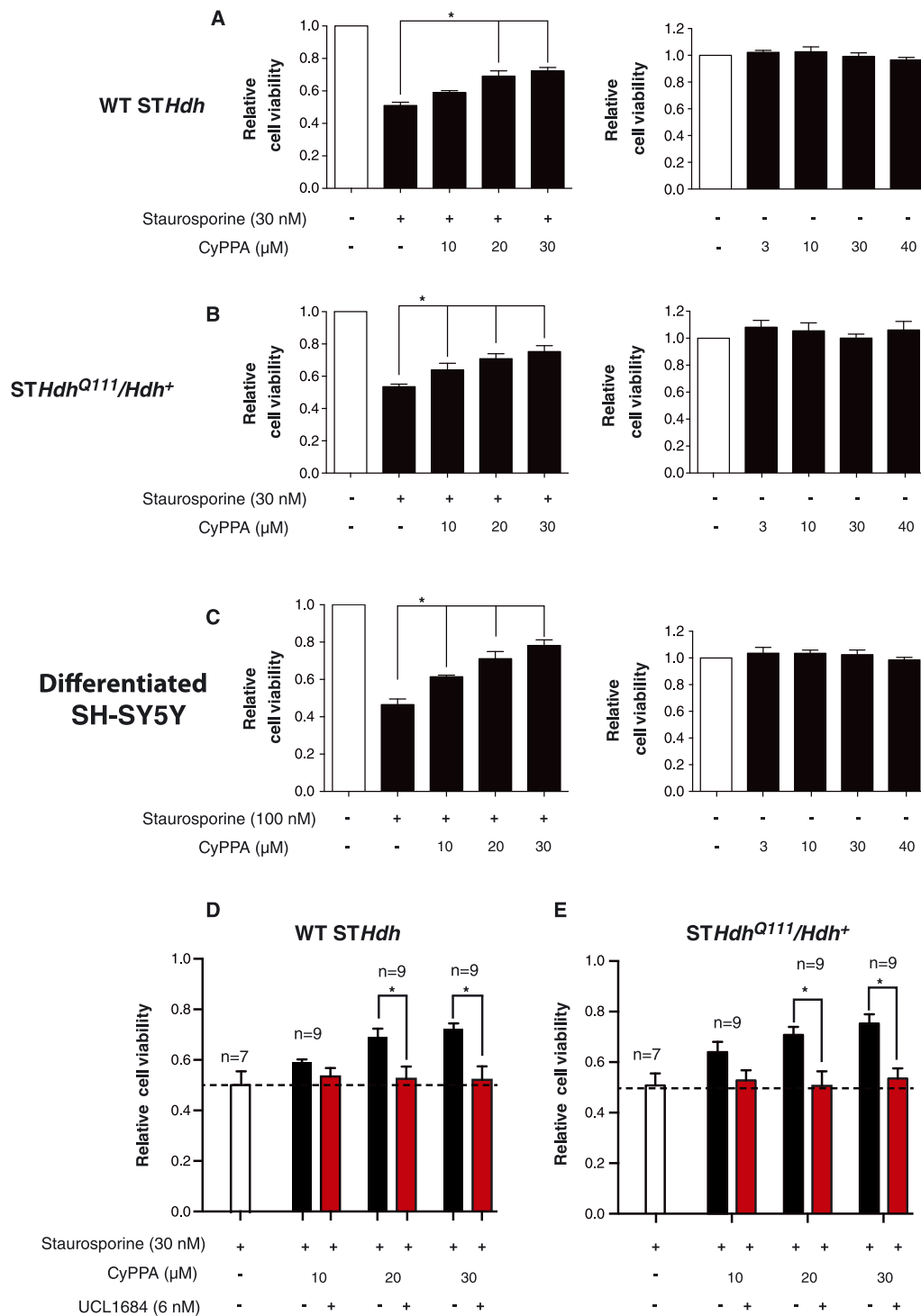


Figure 7

Effect of $K_{Ca2.x}$ channel activation on cell death induced by staurosporine. (A) Effect of $K_{Ca2.x}$ channel activation (with CyPPA) in the presence of staurosporine (30 nM) on wild-type murine striatal neurons (*STHdh*) over 24 h (left panel). (B) Effect of $K_{Ca2.x}$ channel activation (CyPPA) in the presence of staurosporine (30 nM) on heterozygous mutant murine striatal neurons (*STHdh*^{Q111}/*Hdh*⁺) from a murine model of Huntington's disease over 24 h (left panel). (C) Effect of $K_{Ca2.x}$ channel activation (CyPPA) in the presence of staurosporine (100 nM) on differentiated SH-SY5Y cells over 24 h (left panel). CyPPA treatment alone does not affect cell viability in any of the cell lines over this time frame (right panels). Data points represent mean \pm SEM; $n = 6$. * $P < 0.05$, significant difference from staurosporine treatment alone. (D) Effect of UCL1684 on CyPPA-mediated rescue of wild-type murine striatal neurons (*STHdh*) cells from staurosporine treatment. UCL1684-treated cells were not statistically different from staurosporine alone; $n = 7-9$; two-way ANOVA – Tukey's HSD. (E) Effect of UCL1684 on CyPPA-mediated rescue of heterozygous murine striatal neurons (*STHdh*^{Q111}/*Hdh*⁺) cells from staurosporine treatment. UCL1684-treated cells were not statistically different from staurosporine alone; $n = 7-9$; two-way ANOVA – Tukey's HSD.

cancer subtypes and also both endocrine responsive and endocrine-resistant models, overlap perfectly with the IC_{50} values previously reported for these $K_{Ca2.x}$ channel blockers (Dunn, 1999; Strobaek *et al.*, 2006). Such $K_{Ca2.x}$ channel knockdown most likely provides a pro-apoptotic signal, as siRNA-mediated knockdown and pharmacological inhibition were accompanied by a decrease in Bcl-2 expression and a rise in full length and cleaved active caspase-7 and cleaved caspase-9 expression. Furthermore, activation of $K_{Ca2.x}$ channels reduced the effect of an apoptosis inducer, staurosporine, on three CNS-derived cell lines, an effect that could be reversed by blockade of these channels. While we should be mindful of the complex heterogeneity of clinical breast cancer, this provides clear, strong evidence that low MW inhibitors of $K_{Ca2.x}$ channels may represent a novel therapeutic avenue in the treatment of primary ER⁺ and, more importantly, ER⁻ and acquired endocrine-resistant breast cancer.

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Author contributions

Z. A. A. carried out the growth assays, siRNA, RT-PCR and western blotting. C. D. C. generated draft manuscript and carried out statistical analysis. J. M. W. G. coordinated the endocrine-resistant models and microarray data. All authors conceived of the study and participated in its design and coordination and wrote, read and approved the final manuscript.

Conflict of interest

The authors declare no competing financial interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Densitometry of $K_{Ca2.2}$, $K_{Ca2.3}$, Bcl-2 and full length caspase-7 in response to siRNA treatment.

Figure S2 Effect of CyPPA on MCF-7 and MDA-MB-231 cell viability.

Figure S3 Densitometry showing the effect of UCL1684 on expression of apoptotic markers in MCF-7 cells.

Figure S4 Effect of UCL1684 and NS8593 on the viability of a number of cell lines of CNS origin.